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Herpes Simplex Virus VP16, but Not ICP0, Is Required To Reduce Histone Occupancy and Enhance Histone Acetylation on Viral Genomes in U2OS Osteosarcoma Cells▽†

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The herpes simplex virus (HSV) genome rapidly becomes associated with histones after injection into the host cell nucleus. The viral proteins ICP0 and VP16 are required for efficient viral gene expression and have been implicated in reducing the levels of underacetylated histones on the viral genome, raising the possibility that high levels of underacetylated histones inhibit viral gene expression. The U2OS osteosarcoma cell line is permissive for replication of ICP0 and VP16 mutants and appears to lack an innate antiviral repression mechanism present in other cell types. We therefore used chromatin immunoprecipitation to determine whether U2OS cells are competent to load histones onto HSV DNA and, if so, whether ICP0 and/or VP16 are required to reduce histone occupancy and enhance acetylation in this cell type. High levels of underacetylated histone H3 accumulated at several locations on the viral genome in the absence of VP16 activation function; in contrast, an ICP0 mutant displayed markedly reduced histone levels and enhanced acetylation, similar to wild-type HSV. These results demonstrate that U2OS cells are competent to load underacetylated histones onto HSV DNA and uncover an unexpected role for VP16 in modulating chromatin structure at viral early and late loci. One interpretation of these findings is that ICP0 and VP16 affect viral chromatin structure through separate pathways, and the pathway targeted by ICP0 is defective in U2OS cells. We also show that HSV infection results in decreased histone levels on some actively transcribed genes within the cellular genome, demonstrating that viral infection alters cellular chromatin structure.

Herpes simplex virus (HSV) is a double-stranded DNA virus that undergoes productive replication in the nucleus of infected cells. The linear genome is packaged into a nucleocapsid that is released into the cytoplasm upon fusion of the viral and host cell membranes. Also released are the preformed tegument proteins, which play important roles in counteracting host defenses and stimulating viral gene expression. The tegument protein VP16 acts to stimulate immediate-early (IE) gene expression through the recruitment of general transcription factors and RNA polymerase II to the IE promoters (30, 73), launching the temporal cascade of gene expression.

The HSV genome is thought to be complexed with the polyamine spermine within virions (9, 25, 58). Upon injection into the nucleus, the genome associates with host histones (32, 33, 38, 46), most likely in a form involving the four core histones (22, 46), and at a density significantly less than that of cellular chromatin (22, 32, 46). Nucleosomes are the basic repeating units of chromatin comprised of ~146 bp of DNA wrapped around a histone octamer composed of two copies of each of the four core histone proteins (H2A, H2B, H3, and H4). The structure of chromatin can be altered both by post-translational modifications of histones and through ATP-dependent remodeling of the nucleosomes (42).

Chromatin remodeling involves eviction or sliding of nucleosomes along the DNA template, increasing the accessibility of the DNA to other interacting proteins. These processes require specific chromatin remodeling complexes that hydrolyze ATP and are recruited to the DNA through covalent modifications of histones (64), which includes acetylation, methylation, phosphorylation, and ubiquitination. How each histone modification influences gene expression is not yet fully understood (35), but some marks have been generally linked to transcriptional outcomes. For example, histone acetylation correlates with transcriptional activation, and histone methylation correlates with either activation or silencing depending on which residue within the histone is methylated (42). Acetylation is thought to relax the interactions between histones and DNA by altering the net charge of the nucleosome (23) and additionally enhances transcription by recruiting chromatin remodeling complexes (31, 37). Modified histones provide docking sites for proteins that contain specific interaction motifs. For example, bromodomain-containing proteins bind to acetylated histones, while chromodomain-containing proteins, such as the heterochromatin-associated protein HP1, bind to histone H3 trimethylated at lysine 9 (42).

During latent HSV infection the nucleosomes on the viral genome are arranged in a regular repeating pattern similar to cellular chromatin (12). The histones bound to most regions of the genome display features characteristic of transcriptionally silent chromatin, such as reduced acetylation, increased levels

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of H3K9 di- and trimethylation (73) and H3K27 trimethylation (7, 48), and the presence of the histone variant macroH2A (48). The exceptions are the histones found within the promoter and 5′ region of the latency-associated transcript (LAT), which bear activating marks such as acetylation of histone H3 lysine 9 and 14 (H3K9/K14Ac) (43, 44, 73). LATs are the only transcripts expressed in latently infected neurons (68, 69), and these observations therefore suggest that covalent histone modifications play an important role in regulating HSV gene expression during latency.

In contrast to latency, during productive infection the histones are arranged on the viral genome in an irregular, nonrepeating pattern (49, 56). As assessed by chromatin immunoprecipitation (ChIP), histone occupancy is often significantly lower than on the cellular genome (46), although the levels depend on the multiplicity of infection (MOI) used (8). Accumulating evidence suggests that histone modifications may play an important role in regulating HSV gene expression during productive infection (reviewed in references 40 and 47). For example, marks correlating with active transcription, such as H3K9/K14Ac and H3K4 trimethylation, are found within promoters of actively transcribed viral genes (32, 33, 44). The histone methyltransferase Set1 is important in maintaining the H3K4 trimethylation modification on the viral genome, and viral transcription and replication are inhibited when Set1 levels are knocked down by small interfering RNAs (siRNAs). (33). In addition, knocking down the expression of HIRA, a chaperone for the histone H3 variant H3.3, results in decreased association of histone H3.3 with the viral genome and decreased viral transcription and replication (59). These results are consistent with the idea that HIRA actively loads H3.3 onto the viral genome and that viral gene expression is enhanced by this histone variant.

Extensive studies into the mechanism of action of the acidic activation domain (AD) of VP16 in heterologous systems have revealed that it recruits general transcription factors, RNA polymerase II, histone acetyltransferases (HATs), and ATP-dependent chromatin remodeling complexes to promoters (32, 39, 41, 52, 54, 72, 74), and ChIP experiments indicate that the AD also recruits these proteins to viral IE promoters during productive HSV infection (32).

The AD has also been implicated in preventing the deposition, or enhancing the removal, of histones from viral IE promoters (32, 46): histone occupancy is higher on a VP16 AD mutant genome than on the wild-type genome (46). However, the biological significance of these effects of VP16 on viral chromatin structure has been called into question by two recent findings. First, knocking down expression of HATs or chromatin remodeling complexes via siRNAs (either singly or in combination) has no detectable effect on viral IE gene expression in cultured cells (45). Second, HSV-2 superinfection of cells harboring a silent HSV-1 VP16 AD mutant virus activated transcription from the mutant genome without reducing histone levels or increasing histone acetylation (46). This latter observation raises the possibility that once histones are loaded onto the VP16 AD mutant genome they are refractory to subsequent removal or acetylation and suggests that they do not preclude viral gene expression.

The IE protein ICP0 has also been implicated in regulating chromatinization of the viral genome. Cliffe and Knipe demonstrated that the genome of an ICP0-null mutant displays higher levels of bound histone H3 and reduced H3 acetylation compared to wild-type virus during a low-multiplicity infection of HeLa cells (8). Consistent with these results, Ferenczy et al. demonstrated that a viral mutant that expresses only ICP0 displays lower histone occupancy and higher histone acetylation levels than a mutant expressing no viral genes (22). ICP0 has been shown to dissociate histone deacetylases (HDACs) 1 and 2 from the CoREST/REST/HDAC repressor complex (26, 27) and interacts with HDACs 5, 6, and 7 (50), suggesting a direct role in modulating the acetylation of histones bound to the viral genome. Whether these activities contribute to the ability of ICP0 to stimulate transcription of all classes of viral genes (3, 4, 13–15, 24, 36, 57, 62, 65, 70) remains to be determined.

HSV mutants lacking ICP0 or the activation function of VP16 share several phenotypic similarities, including a much higher than normal particle/PFU ratio and lower levels of IE gene expression upon low-multiplicity infection of many cell types (1, 4, 14, 66, 70). The phenotype of such mutants varies between cell types, with the most restrictive being primary human fibroblasts (15). After infection of nonpermissive cells, the viral genome is retained in a transcriptionally silent, extrachromosomal state (1, 15, 29, 30, 34, 60, 61, 63, 65, 70). Such quiescent genomes are actively repressed, as shown by the finding that heterologous promoters embedded within them are also silenced (34, 53, 61, 65). However, quiescent genomes can be efficiently reactivated by ICP0 provided in *trans* (29, 30, 34, 60, 61, 65, 70). The histones bound to quiescent genomes are underacetylated, enriched in H3K9 methylation, and complexed with heterochromatin-associated protein HP1, all classical marks of transcriptional repression (10, 22). After ICP0-mediated derepression, histone acetylation increases markedly (10), suggesting one role of ICP0 in reactivation of quiescent genomes may be to alter histone modifications.

Although VP16 and ICP0 mutants display severely restricted gene expression profiles in many cell types, the activation functions of these proteins are largely dispensable in the human osteosarcoma cell line U2OS (66, 75). We previously provided evidence that the permissive phenotype of U2OS cells stems from defects in an innate antiviral repression mechanism that is present in other cell types (28). We further suggested that the defect might inactivate a chromatin assembly pathway that incorporates newly delivered viral genomes into repressive chromatin. We therefore used ChIP assays to determine whether U2OS cells are competent to load histones onto HSV DNA and, if so, whether ICP0 and/or VP16 are required to reduce histone H3 occupancy in this cell type. Our results clearly indicate that U2OS cells are able to load high levels of underacetylated histones onto viral DNA in the absence of VP16 activation function. However, in contrast to the situation in HeLa cells, ICP0 does not appear to regulate histone H3 levels or acetylation on the viral genome in this cell line.

MATERIALS AND METHODS

Cells and viruses. HeLa cervical carcinoma and U2OS osteosarcoma cells were obtained from the American Type Culture Collection and maintained in Dulbecco modified Eagle Medium supplemented with 10% fetal bovine serum, 50 U of penicillin/ml, and 5 μg of streptomycin/ml.

HSV-1 strains KOS, n212 (lacking the activation function of ICP0 [5]), and

TABLE 1. Real-time PCR primers[a]

Gene region	Orientation	Primer sequence (5′–3′)
GAPDH gene body	F	ACTAGGCGCTCACTGTTCTCTCCCT
	R	AACTCACCCGTTGACTCCGACCTT
U3 promoter	F	GCGCACCACACCAGGAGCAAACA
	R	AGCATAATACTGAATGACAGCCAA TCACAAACT
hsSat3	F	CAATCATCCAACGGAAGCTAATGG AATCAACA
	R	TCCATTCGATGACGAGTCCATCCA TTTCAA
ICP27 TSS	F	CACCACCAGAGGCCATATCCGACA
	R	AGCATATCAATGTCAGTCGCCATG ACCG
ICP27 ORF	F	TGTGCGGCCTGGACGAACTGTGTT
	R	TGGCCAGAATGACAAACACGAAG GATGC
TK promoter	F	CCACACGCGTCACCTTAATATG CGAA
	R	ATTGGCGAATTCGAACACGCAGAT GCAG
VP16 promoter	F	TCGGATTGGGAAACAAAGGCAC GCAA
	R	TCCGTACCCAGACAATAAAGCACC AACAGG

[a] F, forward; R, reverse.

V422 (lacking the activation function of VP16 [66]) were grown on U2OS cells, and titers were determined on the same cell type. V422 stocks were grown in the presence of 3 mM hexamethylene *bis*-acetamide (HMBA; Sigma). Cell-free virus harvested from culture medium was used in all experiments.

Viral infections were performed using standard procedures. At 1 h after infection at 37°C, the cells were washed once with phosphate-buffered saline (PBS) and then twice with a low-pH buffer (40 mM citric acid, 10 mM KCl, 135 mM NaCl [pH 3.0]) for 30 s to inactivate or remove any bound virus that had not penetrated the cells. Cells were then washed twice with growth medium, and growth medium containing 300 μg of phosphonoacetic acid (PAA)/ml was added back to the cultures. For experiments using cycloheximide, 60 μg of the protein synthesis inhibitor/ml was added during at the time of infection and re-added after the acid washes.

In all experiments, the input doses of n212 and V422 were adjusted to give rise to the same levels of viral DNA as 1 PFU of KOS at 3 h postinfection (hpi; in the presence of PAA). The appropriate MOIs (which ranged from 0.875 to 1.1) were determined by infecting cells with 1 PFU/cell of each virus for 3 h and harvesting the total cellular DNA as per the ChIP protocol (up to and including the sonication step; see below). The amount of viral DNA in each sample was then assessed by real-time PCR using primers specific for the ICP27 gene.

ChIP and real-time PCR. ChIP was performed as described previously (8). Briefly, 3×10^6 HeLa or U2OS cells were grown in 100-mm dishes and infected as described above. Cells were fixed by the addition of formaldehyde to a final concentration of 1% for 10 min. Cross-linking was halted by the addition of 125 mM glycine for 5 min. Cells were washed with PBS, resuspended in 1% sodium dodecyl sulfate (SDS) lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris [pH 8.1]), left for 10 min on ice, and diluted threefold with radioimmunoprecipitation assay (RIPA) buffer (0.1% SDS. 1% sodium deoxycholate, 150 mM NaCl, 10 mM Na_2PO_4, 2 mM EDTA, 1% NP-40). Samples were sonicated with a model 550 Fisher Scientific sonic dismembrator using 20-s pulses to obtain DNA fragmented to 200 to 500 bp. Samples were diluted threefold with RIPA buffer and precleared with protein A-agarose/salmon sperm DNA (Millipore) for 1 h. Protein-DNA complexes were immunoprecipitated with 5 μg of anti-histone H3 antibody (ab1791; Abcam) or 10 μg of anti-acetyl-histone H3 antibody (Millipore catalog no. 06-599) overnight. Immune complexes were collected by incubation with protein A-agarose/salmon sperm DNA for 30 min, followed by washes with low-salt (150 mM NaCl, 20 mM Tris [pH 8.1], 2 mM EDTA, 1% Triton X-100, 0.1% SDS), high-salt (500 mM NaCl, 20 mM Tris [pH 8.1], 2 mM EDTA, 1% Triton X-100, 0.1% SDS), and LiCl (0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris [pH 8.1]) buffers and then two washes with TE (10 mM Tris [pH 7.5], 1 mM EDTA). Protein-DNA complexes were eluted in 200 μl of elution buffer (1% SDS, 0.1 M $NaHCO_3$) warmed to 65°C, rotated for 15 min at room temperature, incubated for 15 min at 65°C, and rotated for another 15 min at room temperature. Eluates were incubated at 65°C for at least 4 h in the presence of 200 mM NaCl and 1 μg of RNase A. DNA was isolated by using a Qiagen PCR purification kit and eluted in 100 μl of H_2O.

PCRs (10 μl) were prepared by using 2 μl of DNA, 1.6 μM concentrations of each primer (Table 1), and 2× Sybr green Mix (20 mM Tris [pH 8.3], 100 mM KCl, 6 mM $MgCl_2$, 1.6% glycerol, 0.02% Tween 20, 4% dimethyl sulfoxide, 0.4 mM concentrations of deoxynucleoside triphosphates [each], 0.06 U of Platinum *Taq*/μl, 1× Sybr green). The specificity of each primer pair was analyzed by determining the melting curves for each PCR product. Samples were analyzed in duplicate, and relative copy numbers were determined by comparison with a standard curve generated by a 10-fold dilution series of pooled input samples.

As detailed in Results, we found that HSV infection reduces histone occupancy at the cellular GAPDH (glyceraldehyde-3-phosphate dehydrogenase) locus, a result that precluded using GAPDH as an internal control for experiment-to-experiment variation. Therefore, in order to obtain independent replicates that minimize variation due to use of different batches of reagents or changes in condition of the sonicator probe, we routinely performed three independent replicates of each experiment over the course of 1 week.

Northern blotting. Cells were grown to confluence in 60-mm dishes and infected exactly as described above for ChIP experiments. After the indicated times, total RNA was harvested with TRIzol (Invitrogen) and prepared by using standard procedures. Then, 10 μg of RNA was run on a denaturing agarose gel, blotted, and probed for the ICP27 transcript. Blots were then stripped and reprobed for TK and VP16.

RESULTS

Gene expression profiles of n212 and V422 infection in HeLa and U2OS cells. Previous studies documenting the roles of VP16 and ICP0 in the regulation of HSV genome chromatinization were conducted in HeLa cells (8, 32, 46). We sought to examine the effects of VP16 and ICP0 on genome chromatinization in U2OS cells, which "complement" the growth defect of HSV ICP0 and VP16 mutants (55, 66, 75). In order to provide a basis for assessing the functional significance of any such effects, we first compared the consequences of inactivating ICP0 and VP16 on viral gene expression in U2OS and HeLa cells. To this end, we monitored the accumulation of mRNAs derived from the viral ICP27 (IE), TK (early), and VP16 (leaky-late) genes after infection of U2OS and HeLa cells with 1 PFU/cell of wild-type KOS, the ICP0 mutant n212, and the VP16 AD mutant V422 (Fig. 1). Viral DNA replication was blocked by the addition of the viral DNA polymerase inhibitor PAA at the time of infection in order to restrict the analysis to expression from input viral genomes.

In HeLa cells, ICP27 mRNA accumulated between 3 and 6 hpi with both KOS and n212, whereas virtually no ICP27

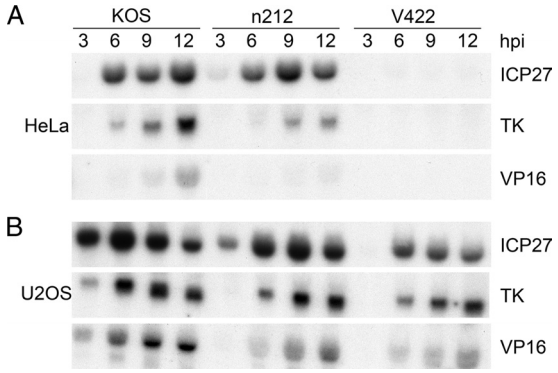

FIG. 1. Gene expression profiles of KOS, n212, and V422 in HeLa and U2OS cells. HeLa and U2OS cells were infected with the indicated viruses in the presence of PAA as described in Materials and Methods. Total RNA was harvested after the indicated lengths of time and subjected to Northern blot analysis using probes for ICP27, TK, and VP16.

mRNA was detected over the 12-h time course with V422 (Fig. 1A). n212 displayed wild-type levels of ICP27 mRNA, but TK and VP16 transcripts accumulated with delayed kinetics and to lower levels. The results with TK mRNA are similar to the previous findings of Jordan and Schaffer (36), who examined accumulation of viral mRNAs during n212 infection of Vero cells (ICP27 and VP16 mRNA were not examined in that study). In contrast, TK and VP16 mRNA could not be detected during infection with V422. Thus, both mutants exhibit a restricted gene expression profile in HeLa cells, with V422 displaying a much more severe defect.

As expected, U2OS cells supported much higher levels of viral gene expression than HeLa cells after infection with n212 and V422 (Fig. 1B). However, significant defects relative to wild-type KOS were nevertheless observed. For example, abundant ICP27 mRNA was present by 3 hpi with KOS, but accumulation of the transcript was delayed with n212 and V422, and the levels were significantly reduced at all time points with V422 (Fig. 1B). Similarly, accumulation of TK and VP16 mRNAs was delayed and reduced with both mutants. Overall, these data indicate that V422 is somewhat more impaired than n212 in this cell type, as assayed by gene expression in the presence of PAA.

Histone H3 occupancy on the n212 and V422 genomes in HeLa and U2OS cells. We previously suggested that the recessive defect that renders U2OS cells permissive to VP16 and ICP0 mutants might inactivate a chromatin assembly pathway that represses incoming viral genomes in other cell types (28). To test this hypothesis, we used ChIP assays to monitor the loading and acetylation of histone H3 on viral genomes in HeLa and U2OS cells infected with wild-type KOS, n212 and V422. All experiments were performed in the presence of PAA to restrict the analysis to input viral genomes. In addition, the n212 and V422 input multiplicities were adjusted to deliver the same amount of input viral DNA (measured at 3 hpi) as is achieved with 1 PFU/cell of wild-type KOS (Materials and Methods). In no case did this adjustment alter the MOI by >25%.

We first monitored histone H3 levels at the transcriptional start site (TSS) of the IE ICP27 gene (Fig. 2A). In HeLa cells infected with wild-type KOS, histone H3 levels rose between 1 and 3 hpi and then declined by 6 hpi; in contrast, H3 continued to accumulate on the n212 and V422 genomes, leading to significantly higher levels than on the KOS genome by 6 hpi, a finding consistent with previous reports (8, 32, 46). U2OS cells displayed a very different pattern: H3 levels remained low on the KOS and n212 genomes over the entire time course, while H3 accumulated on the V422 genome (Fig. 2A). These results indicate that the AD of VP16 is required to reduce histone occupancy on the ICP27 TSS in both HeLa and U2OS cells, whereas ICP0 is dispensable in U2OS cells. In addition, the accumulation of high levels of histone H3 on the V422 genome in U2OS cells documents that these cells are competent to assemble histones onto incoming viral genomes. Of note, we consistently observed a higher apparent histone H3 load at the ICP27 TSS in U2OS cells than in HeLa cells, an effect that was also observed at other loci in the HSV genome (Fig. 2B and C). A similar difference in apparent H3 load between HeLa and U2OS cells was also observed at several loci in the cellular genome (data not shown) and thus is not specific to HSV DNA. The basis for this difference remains to be determined.

We next tested whether the increase in histone occupancy at the ICP27 TSS observed with n212 and V422 in HeLa cells, and with V422 in U2OS cells, also occurs at other regions of the viral genome. In HeLa cells, enhanced histone H3 levels were observed at the TK and VP16 promoters with both mutants (Fig. 2B and C), data that are in accord with recent reports documenting that H3 levels are increased at multiple gene loci, including early and late genes, when either VP16 or ICP0 is inactivated (8, 46). Similarly, in U2OS cells, n212 and V422 displayed low and high histone H3 levels, respectively, at all three loci. Thus, the effects of VP16 and ICP0 on histone occupancy are not restricted to IE genes in either cell type.

VP16 is required for enhanced acetylation of histone H3 bound to the viral genome in U2OS cells, whereas ICP0 is dispensable. Cliffe and Knipe demonstrated that the histone H3 bound to the genome of an HSV-1 ICP0-null mutant displays lower levels of acetylation than that bound to the corresponding marker rescue virus during low-MOI infection of HeLa cells, suggesting that ICP0 plays an important role in stimulating histone acetylation in this cell type (8). In addition, Herrera and Triezenberg documented that VP16 stimulates acetylation of histones bound to viral DNA in HeLa cells (32). We therefore sought to determine whether ICP0 and/or VP16 are required for enhanced histone acetylation in permissive U2OS cells.

HeLa and U2OS cells were infected with KOS, n212, or V422 for 6 and 9 h and then harvested for ChIP. Sonicated lysates were divided, and immunoprecipitations were performed with the total histone H3 and acetyl-H3 antibodies in parallel. The quantity of acetylated histones immunoprecipitated from each sample was then normalized to the amount of immunoprecipitated total histone H3. In HeLa cells the levels of acetylated histone H3 were significantly higher on the KOS genome than on n212 or V422 at 6 and 9 hpi for all regions of the viral genome tested (Fig. 3A), confirming previous reports (8, 32). Similar results were observed at 3 hpi (see Fig. S1 in the supplemental material). In contrast, in U2OS cells histone H3 acetylation levels were high for both KOS and n212, but acetylation remained low on the V422 genome (Fig. 3b and see Fig. S1 in the supplemental material). These trends were observed at all regions of the viral genome tested (Fig. 3). In the series of experiments depicted in Fig. 3, n212 displayed somewhat higher levels of histone acetylation than KOS. However, this difference was not statistically significant except for the VP16 promoter at 6 hpi, and it was not observed in an independent series of experiments conducted several months later (see Fig. S1 in the supplemental material). These observations indicate that in U2OS cells, histone H3 acetylation requires the activation function of VP16 but does not require the action of ICP0.

Effects of viral infection on histone H3 levels on the cellular genome. During the course of the experiments described above, we also measured histone H3 levels associated with the transcribed body of the cellular GAPDH gene, with the intention of using this value as an internal control to correct for differences in cross-linking and immunoprecipitation efficiencies between experiments. However, the results demonstrated that the histone H3 levels on the GAPDH gene significantly decrease during wild-type HSV infection, in both HeLa and

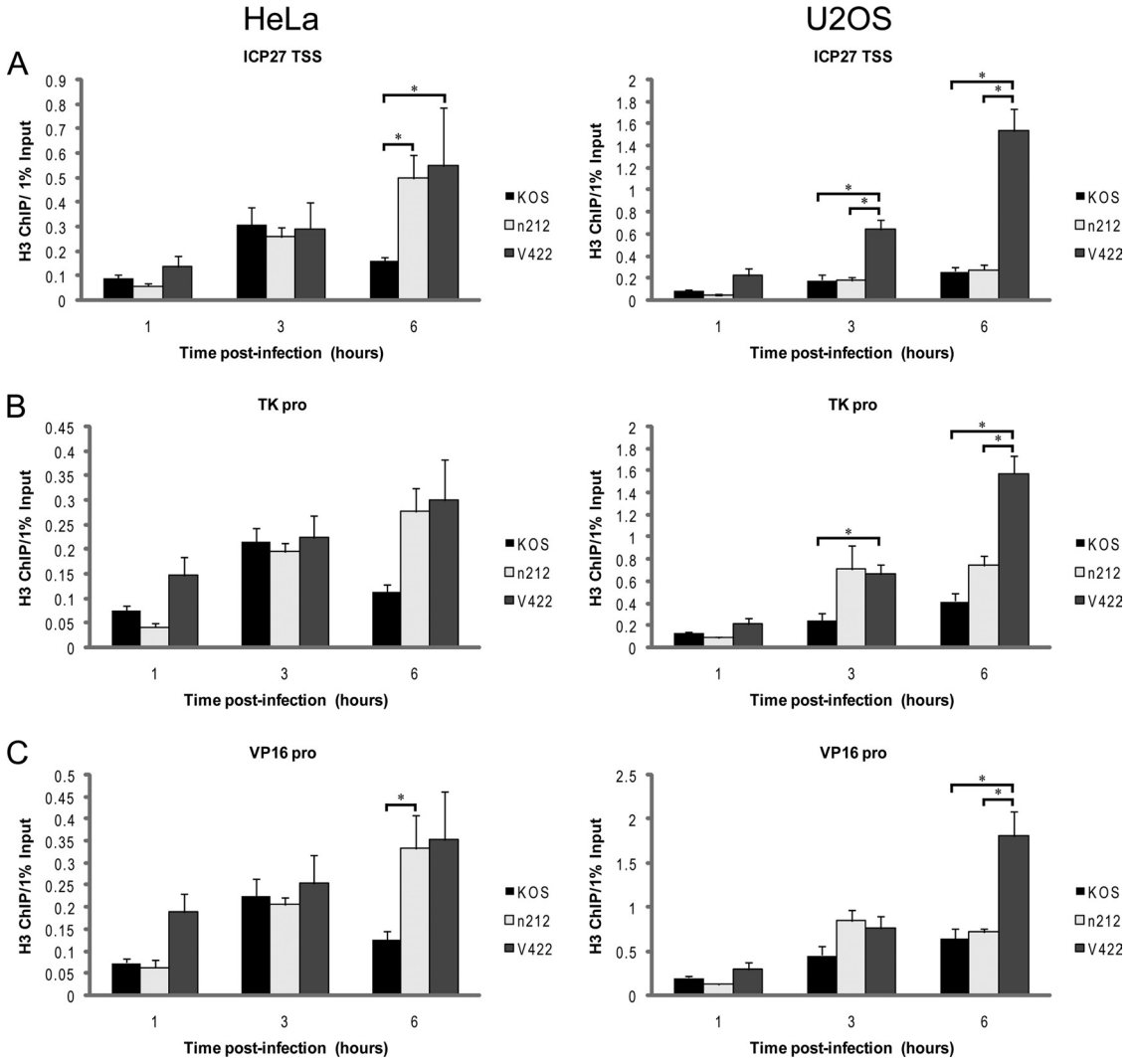

FIG. 2. Histone H3 occupancy on KOS, n212, and V422 genomes in HeLa and U2OS cells. HeLa and U2OS cells were infected with the indicated viruses in the presence of PAA as described in Materials and Methods. Cell lysates were prepared at 1, 3, and 6 hpi. The amount of immunoprecipitated DNA from the ICP27 TSS (A), TK promoter (B), or VP16 promoter (C) was determined. Pooled data from three independent experiments are presented. Samples with mean values that differed significantly ($P < 0.05$, paired Student t test) are indicated (*).

U2OS cells (the data obtained in U2OS cells are shown in Fig. 4A). A similar decrease in histone occupancy was noted after infection with n212 but not after infection with V422 (Fig. 4A). These results were initially surprising, because no such histone depletion has been observed in similar experiments using another set of GAPDH real-time PCR primers at either a low (8) or a high (K. Bryant and D. Knipe, unpublished data) MOI. However, we have recently become aware that the primers used in those studies are specific for one or more GAPDH processed pseudogenes and generate an amplimer of a size consistent with sequences from a pseudogene (A. Cliffe and D. Knipe, unpublished results). In contrast, the primers used in the present report include intron sequences and therefore specifically detect the functional GAPDH gene.

To test whether this effect is specific to GAPDH, we also assessed histone occupancy at the promoter for the cellular U3 snoRNA gene (Fig. 4B) and the pericentric satellite sequence Sat3 (Fig. 4C). A significant decrease in histone occupancy was observed at the U3 promoter during KOS and n212 infection, although no change was detected at the Sat3 region with any virus. Collectively, these data raise the possibility that histone depletion is limited to actively transcribed cellular loci.

To determine whether protein synthesis is required for the observed decreases in histone occupancy, ChIP experiments were performed in U2OS cells infected with KOS, n212 and V422 for 6 h in the presence or absence of cycloheximide. Cycloheximide treatment increased histone occupancy on the GAPDH gene in cell infected with KOS and n212, whereas histone H3 levels remained high with V422 (Fig. 4D). Similar results were obtained in HeLa cells (data not shown).

These results indicate that HSV infection depletes histone H3 from specific regions of the cellular genome and that this process does not require ICP0. Whether this effect represents a cellular response to infection or a specific effect of one or more viral proteins has not yet been determined.

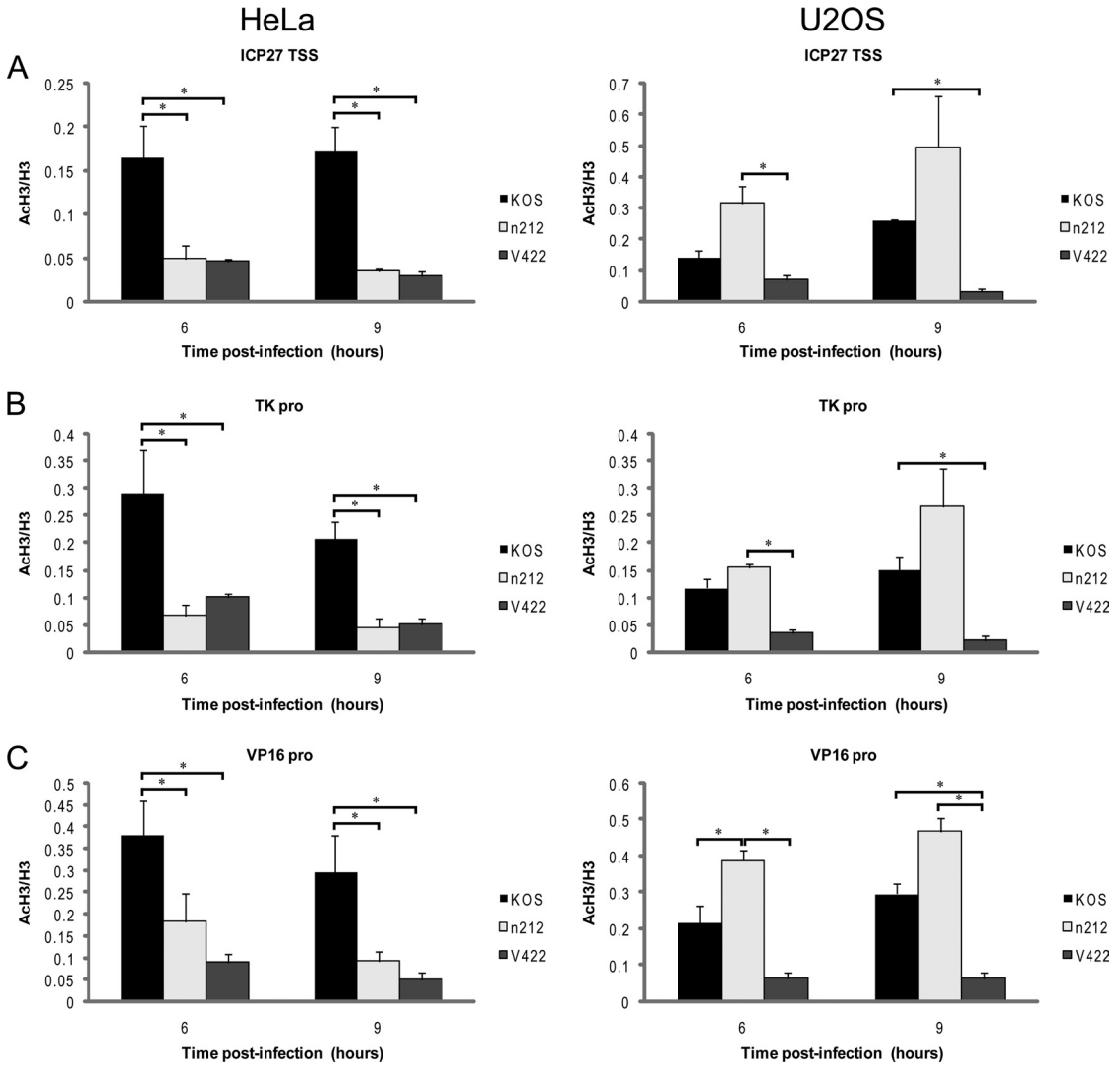

FIG. 3. Acetylated histone H3 on KOS, n212, and V422 genomes in HeLa and U2OS cells. HeLa and U2OS cells were infected with the viruses in the presence of PAA as described in Materials and Methods. Cell lysates were prepared at 6 and 9 hpi. The proportion of acetylated histone H3 associated with ICP27 TSS (A), TK promoter (B), and VP16 promoter (C) viral DNA was determined as the fraction of DNA associated with acetylated H3 normalized to the fraction of DNA associated with total histone H3. Pooled data from three independent experiments are presented. Samples with mean values that differed significantly ($P < 0.05$, paired Student t test) are indicated (*).

DISCUSSION

Previous studies have shown that HSV-1 mutants lacking the activation functions of VP16 or ICP0 accumulate high levels of underacetylated histones at multiple regions of the viral genome in HeLa cells (8, 22, 32, 46). One interpretation of the data presented in those reports is that VP16 reduces histone occupancy and enhances acetylation at IE regions, while ICP0 subsequently acts to produce the same effect over the rest of the viral genome. The effects of VP16 and ICP0 on histone occupancy and acetylation are consistent with the hypothesis that these proteins stimulate viral gene expression at least in part by countering the formation of a repressive chromatin structure. However, as reviewed in the introduction, evidence contrary to this hypothesis has recently been presented by Kutluay et al. (45, 46). Given these observations, the role of chromatin structure in regulating viral gene expression during productive infections clearly requires further investigation.

As one means of exploring the role of histone loading and acetylation in regulating viral gene expression, we examined these processes in U2OS cells, which "complement" the growth defects of both ICP0 and VP16 AD mutants (66, 75). This permissive phenotype is recessive in somatic cell hybrids, indicating that U2OS cells lack an antiviral mechanism that is critical for limiting viral gene expression in restrictive cell types (28). If ICP0 and/or VP16 function to stimulate viral gene expression primarily by preventing the assembly of repressive chromatin on the viral genome, then U2OS cells would likely display a defect in this process. Consistent with this hypothesis, we found that the ICP0 mutant n212 displays a chromatin phenotype similar to that of wild-type virus in U2OS cells: multiple

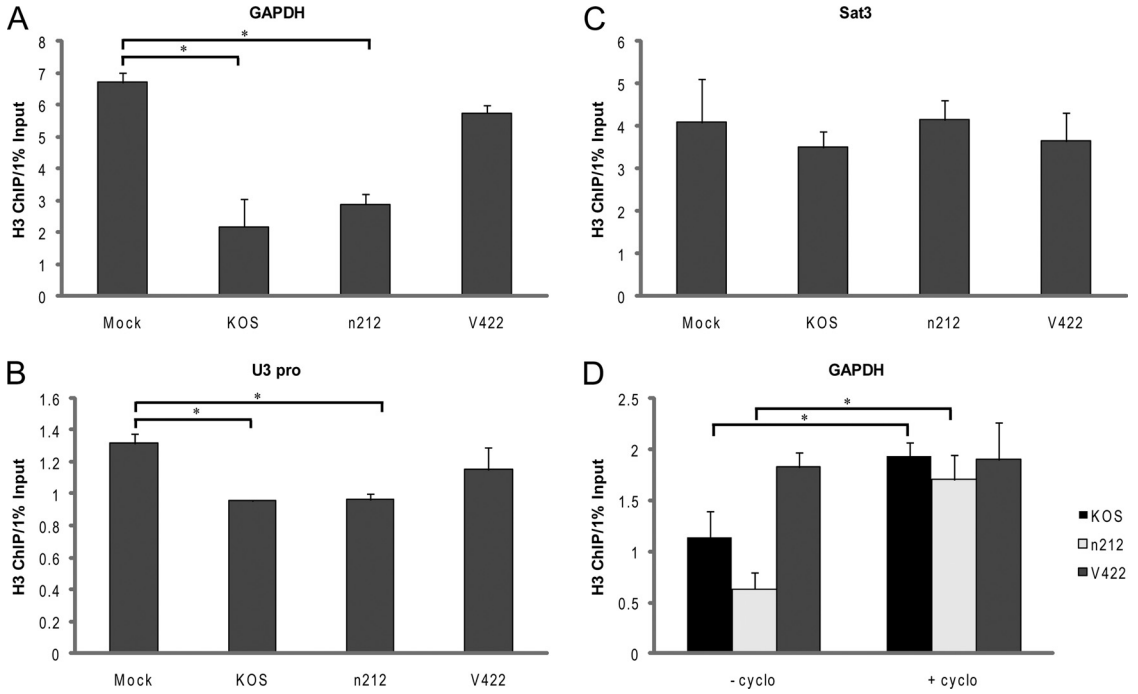

FIG. 4. Effects of HSV infection on histone H3 occupancy at selected regions of the cellular genome. U2OS cells were infected with KOS, n212, or V422 or else mock infected in the presence of PAA as described in Materials and Methods. Cell lysates were prepared at 6 hpi. The amount of immunoprecipitated DNA associated with the GAPDH gene (A), U3 promoter (B), or Sat3 sequence (C) was determined. (D) Infections at 6 h were also performed in the presence of cycloheximide, and the amount of GAPDH DNA immunoprecipitated was determined. Samples with mean values that differed significantly ($P < 0.05$, paired Student t test) are indicated (*). The data displayed in panels A to C were pooled from three independent experiments performed over the course of approximately 1 week; the data displayed in panel D were pooled from a separate set of three independent experiments performed approximately 1 month later.

regions of the genome were associated with low levels of highly acetylated histone H3. Thus, ICP0 is not required to reduce histone occupancy and enhance histone acetylation on the viral genome in this cell line, a finding that is in keeping with the idea that these cells lack a chromatin-based repression mechanism that is targeted by ICP0. However, the VP16 AD mutant V422 behaved very differently: its genome was loaded with high levels of underacetylated histones, just as it is in HeLa cells. This result indicates that U2OS cells are competent to load underacetylated histones onto viral DNA and leads to the surprising conclusion that the VP16 AD acts, either directly or indirectly, to reduce histone occupancy and enhance histone acetylation at multiple regions of the viral genome, in a process that does not require ICP0.

How does the VP16 AD reduce histone occupancy and enhance acetylation on early and late genes? Kutluay and Triezenberg (46) suggested that the accumulation of high levels of underacetylated histones on VP16 mutant genomes is a consequence of reduced transcription, rather than loss of VP16 function *per se*. However, V422 and n212 accumulate TK and VP16 mRNAs with roughly similar kinetics in U2OS cells (Fig. 1) and yet display very different patterns of histone loading and acetylation (Fig. 2 and 3), arguing that reduced transcription is not the cause of the increased histone occupancy at the TK and VP16 loci of V422.

Similarly, the increase is probably not due to the failure to express a downstream viral protein, since V422 replicates quite efficiently in U2OS cells (55, 66), indicating that most or all viral genes are eventually expressed in these cells.

These considerations suggest that VP16 likely acts directly (either on its own or in concert with other viral proteins) to reduce histone loading and enhance histone acetylation at multiple regions of the genome. For example, the VP16 AD might bind and sequester one or more key proteins required for efficient accumulation of underacetylated histones on the viral genome (such as an HDAC or a specialized histone chaperone), or it might actively recruit HATs to multiple regions of the viral genome, in addition to acting specifically at IE promoters. Further studies are required to test this hypothesis.

What is the primary defect in U2OS cells? Our data argue that ICP0 and VP16 are both required to reduce histone occupancy and enhance acetylation at multiple loci on the viral genome in restrictive HeLa cells. The requirement for ICP0 is relieved in permissive U2OS cells, but the requirement for VP16 is maintained. These findings provide strong evidence that ICP0 and VP16 achieve their effects on viral chromatin structure though separate pathways. One model to account for these findings is that two independent chromatin assembly pathways are capable of loading high levels of underacetylated histone onto the viral genome. One of these is inactivated by ICP0, whereas the other is modulated by VP16. Under this scenario, U2OS cells lack the pathway targeted by ICP0 but retain the pathway that is targeted by VP16.

It is tempting to speculate that the chromatin assembly path-

way targeted by ICP0 is linked to the well-documented ND10-based antiviral repression system. One of the earliest cellular responses to HSV infection is the mobilization of ND10 components to the incoming viral genome (17, 21, 51, 67). A critical role of ICP0 is to block the recruitment of ND10 components to the incoming viral genome through the proteasome-dependent degradation of SUMO-modified forms of PML and Sp100 (2, 6, 16, 17, 19, 20). The ability of ICP0 to cause degradation of ND10 components closely correlates with its ability to stimulate viral gene expression and reactivate quiescent genomes (20). Consistent with a link between ND10 components and chromatin assembly, the HIRA histone chaperone localizes to ND10 (76), and quiescent HSV genomes bearing high levels of underacetylated histones (10, 22) are surrounded by a shell of PML and other ND10 components (18). However, it is important to note that HDAC inhibitors such as trichostatin A are unable to fully replace ICP0 in stimulating gene expression in restrictive human fibroblasts (19), and the portion of ICP0 that interacts with CoREST and causes the translocation of HDACs 1 and 2 to the cytoplasm is not required for ICP0-induced activation of viral gene expression (20). Thus, the underacetylated histones that accumulate on the viral genome in the absence of ICP0 may not be directly responsible for primary genome repression. Perhaps loading of underacetylated histones is linked to, but downstream of, the ND10-based genome detection and primary repression system. Consistent with this interpretation, increased chromatinization of the n212 genome was most evident at 6 hpi in our experiments (Fig. 2), but genome repression has been detected in fluorescence-based assays at much earlier times in other cell types (15). Thus, it is possible that U2OS are defective in the ND10-based detection system, rather than in histone acetylation and removal *per se*. Further studies are required to test this hypothesis.

If, as argued above, two pathways exist to load high levels of underacetylated histone onto the viral genome, then two key questions arise. First, are the two pathways linked to functionally distinct outcomes? Second, what factors control which pathway predominates in cells where both are potentially operative? Further studies are required to address these questions.

Increased levels of underacetylated histone H3 on the viral genome do not preclude viral gene expression. Our data demonstrate that viral gene expression profiles do not always strictly correlate with the load and acetylation status of histone H3 on the viral genome. For example, in HeLa cells both n212 and V422 display higher levels of underacetylated histone H3 than wild-type virus at all loci tested (Fig. 2 and 3), but n212 expresses high levels of ICP27 mRNA, whereas V422 is essentially silent (Fig. 1). Similarly, V422 displays an increased load of underacetylated histone H3 in U2OS cells, where significant viral gene expression is observed. These data indicate that the increased levels of underacetylated histones that accumulate on the viral genome in the absence of ICP0 and/or the VP16 AD do not present an insuperable barrier to viral gene expression. Kutluay and Triezenberg recently reached the same conclusion (46). Although it might be argued that only a small pool of unchromatinized viral genomes are actively transcribed, Kutluay and Triezenberg's data demonstrate that RNA polymerase II and the histones H3 and H2A co-occupy the same DNA templates, suggesting that at least some of the transcribed RNA comes from highly chromatinized and underacetylated genomes (46).

It is important to stress that the increased levels of histone H3 found on the viral genome in U2OS cells in the absence of VP16 activation function are similar to, or lower than, the histone density at two actively transcribed cellular genes (compare Fig. 2 and 4) and thus clearly do not by themselves preclude transcription. What is surprising is that histone acetylation does not appear to be required for viral gene expression. On the cellular genome, histone acetylation levels are generally high at promoters of actively transcribed cellular genes and are thought to recruit chromatin remodelers which stimulate transcription (23, 31, 37). In contrast, although the VP16 AD recruits HATs and chromatin remodeling complexes to viral IE promoters (32), these factors are not required for efficient IE transcription during productive infection (45). It is currently unknown why VP16 recruits HATs and remodeling complexes to IE promoters; perhaps these factors are important during VP16-mediated reactivation from latency (71).

Effects of HSV infection on cellular chromatin structure. Our finding that HSV infection results in a reduced histone H3 load on portions of the cellular *GAPDH* and *U3* genes indicates that HSV gene products can affect chromatin structure on at least some cellular genes, much as they do on the viral genome. HSV affects a wide variety of cellular processes, such as inhibiting host gene transcription; however, these results are the first to show that cellular chromatin can also be affected. The mechanisms involved have yet to be defined. Although ICP0 is not required, newly synthesized viral (or cellular) proteins are needed. It has previously been demonstrated that the linker histone H1 is mobilized upon HSV infection in a manner dependent on IE or E gene expression (11). One possible interpretation of these results is that histones are mobilized away from cellular chromatin and onto the incoming viral genomes, although this has not been directly demonstrated. The histone variant H3.3 becomes associated with the viral genome at early times postinfection using a mechanism involving the DNA replication-independent histone chaperone HIRA (59). It is not clear whether HIRA draws from H3.3 bound to the cellular genome, but cycloheximide treatment increases histone occupancy on the viral genome in HeLa cells (46), suggesting that new synthesis of histone H3.3 is likely not required.

Our results also have implications for the choice of internal control sequences for ChIP experiments conducted on HSV-infected cells. An internal control sequence is routinely used in these protocols to control for variability in the efficiency of chromatin immunoprecipitation between experiments. In HSV studies, this has often been a cellular gene sequence. The results presented here document that the specific regions of the *GAPDH* and *U3* genes detected by the primers used in the present study cannot be used as normalization controls. However, no change in histone H3 load was observed with the Sat3 sequences and, as noted above in other studies (8), we have not observed a change in histone occupancy on cellular sequences in a *GAPDH* processed pseudogene. Therefore, certain sequences such as those detected with primers for the Sat3 sequences or the GAPDH pseudogene provide good internal controls for normalization of ChIP efficiency in different sam-

ples. These results argue that care must be taken in the choice of the host genomic sequences used for normalization in ChIP studies involving HSV infection. In any case, it will be interesting to determine whether HSV depletes histones only from actively transcribed cellular genes as part of its lytic replication process.

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